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Review

Fighting disease by selective autophagy of aggregate-prone proteins

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ABSTRACT

Ubiquitinated protein aggregates are hallmarks of a range of human diseases, including neurodegenerative, liver and muscle disorders. These protein aggregates are typically positive for the autophagy receptor p62. Whereas the ubiquitin-proteasome system (UPS) degrades shortlived and misfolded ubiquitinated proteins that are small enough to enter the narrow pore of the barrel-shaped proteasome, the lysosomal pathway of autophagy can degrade larger structures including entire organelles or protein aggregates. This degradation requires autophagy receptors that link the cargo with the molecular machinery of autophagy and is enhanced by certain posttranslational modifications of the cargo. In this review we focus on how autophagy clears aggregate-prone proteins and the relevance of this process to protein aggregate associated diseases.

1. Introduction

Accumulation of ubiquitinated protein aggregates accompanies several neurodegenerative disorders, as well as a number of other protein aggregation diseases (proteinopathies) including those affecting muscles, heart and liver. These aggregates consist of misfolded or aggregate-prone mutated versions of normal proteins, exemplified by the cytotoxic polyglutamine-expanded huntingtin protein causing Huntington's disease (HD). A well-characterized pathway for the cell to degrade misfolded proteins is through the ubiquitin-proteasome system (UPS), where misfolded proteins are tagged with ubiquitin and targeted to the proteasome for degradation. However, aggregate-prone proteins are poor substrates for proteasomal degradation as proteins must pass through the narrow barrel-shaped proteasome to be degraded [1,2]. Recent studies have shown that also autophagy, a lysosomal degradation pathway for intracellular material, can degrade misfolded proteins. There are three different types of autophagy: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. CMA involves direct import of cytosolic proteins that contain a pentapeptide motif recognized by a chaperone leading to translocation into the lysosomes [3]. Microautophagy involves direct uptake of cytoplasmic components by invagination of the lysosomal membrane [4]. Macroautophagy (hereafter referred to simply as autophagy) involves expansion of a phagophore membrane which engulfs cytoplasmic material as it closes to form a double-membrane vesicle, the autophagosome (Fig. 1). After its formation, the autophagosome can mature by fusion with endosomes to form amphisomes prior to fusion with lysosomes or fuse directly with lysosomes to form the degradative autolysosomes. There the originally cytoplasmic contents are degraded and the resulting macromolecules are released to be reused by the cell. The core molecular machinery of macroautophagy was first elucidated by elegant genetic studies in yeast [4,5] and has later been progressively characterized in mammals. The proteins found in the initial screens are named autophagy-related (Atg) proteins. Although the core autophagy machinery is well conserved from yeast to mammals, differences exist and this review will mainly focus on mammalian autophagy. Autophagy is induced by stresses like starvation, to make the cell recycle its contents in attendance of replenished nutrient supplies. In addition to this

Abbreviations: Aβ, amyloid-β; AD, Alzheimer disease; Alfy, autophagy-linked FYVE protein; ALIS, aggresome-like inducible structure; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; Atg, autophagy-related; Bchs, blue cheese; CMA, chaperone-mediated autophagy; COPI, coat protein complex I; CBP, CREB-binding protein; Cvt, cytoplasm to vacuole targeting; DOR, diabetes- and obesity-regulated gene; ESCRT, endosomal sorting complex required for transport; FTD, frontotemporal dementia; HD, Huntington's disease; HDAC6, Histone deacetylase 6; IF, intermediate filament; LC3, MAP1 light chain 3; LIR, LC3-interacting region; MDB, Mallory-Denk body; MTOC, microtubule-organizing centre; mTOR, mammalian Target of Rapamycin; MVB, multivesicular body; NBR1, neighbour of BRCA1 gene 1; NES, nuclear export signal; NLS, nuclear localization signal; PD, Parkinson's disease; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol 3-kinase; PI3P, phospatidylinositol-3-phosphate; PML, promyelocytic leukemia; ROS, reactive oxygen species; SCA1, spinocerebellar ataxia 1; TDP-43, TAR DNA binding protein-43; UBA, ubiquitin-associated domain; ULK, unc-51-like kinase; UPS, ubiquitin-proteasome system

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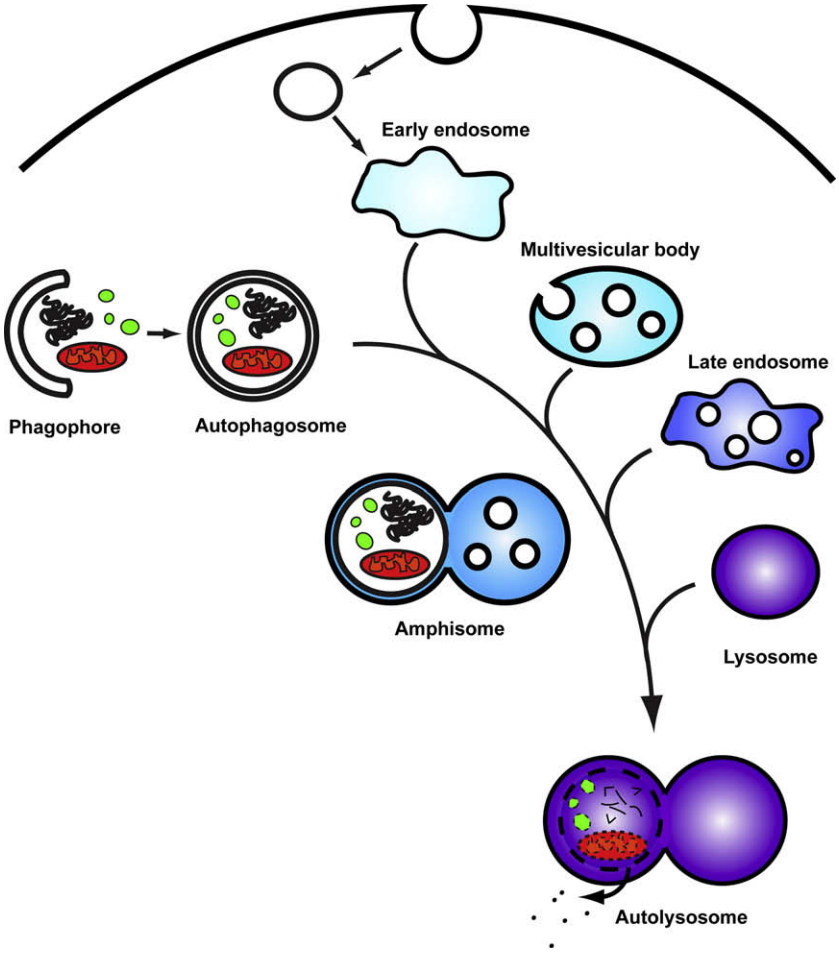

Fig. 1. Autophagy in mammalian cells. When autophagy is induced a phagophore membrane nucleates and expands to form an autophagosome, thereby engulfing part of the cytoplasm, including cytosolic proteins, organelles and aggregated proteins. The autophagosome may fuse directly with lysosomes or mature by fusion with different endocytic compartments (early endosomes, multivesicular bodies and late endosomes) to form amphisomes containing both autophagosomal and endosomal content. The early endosome is a sorting compartment from where internalized cargo like nutrient receptors recycles back to the plasma membrane, whereas cargo destined for degradation is sorted into the intraluminal vesicles of forming multivesicular bodies (MVBs). These MVBs then mature into late endosomes, which fuse with lysosomes for the final degradation of the internalized contents. This stepwise maturation of the endocytic compartments is associated with decreasing pH throughout the pathway. Fusion of autophagosomes with each of these endocytic compartments also supports a stepwise fusion model of autophagosome maturation. Finally the autophagosomes or amphisomes fuse with lysosomes where the originally cytoplasmic material becomes degraded and the resulting macromolecules recycled back to the cytosol to be reused by the cell.

direct pro-survival effect, over the past years it has been discovered that autophagy plays essential roles in development, immunity and prevention of disease [6,7].

2. A brief introduction to the molecular core machinery of autophagy

The mammalian autophagic machinery has already been extensively reviewed [8,9] and only the main components are described here. In brief, the nucleation and expansion of autophagic membranes requires four groups of proteins: (1) the Atg1/unc-51-like kinase (ULK) complex, (2) the Vps34/class III phosphatidylinositol 3-kinase (PI3K) complex I, which forms phospatidylinositol-3-phosphate (PI3P), (3) the transmembrane protein mAtg9 and its associated cycling machinery and (4) the two ubiquitin-like proteins Atg12 and Atg8/MAP1 light chain 3 (LC3) and their conjugation systems.

2.1. Nucleation

The induction of autophagy is directly inhibited by the mammalian Target of Rapamycin (mTOR) complex, which coordinates cell growth and autophagy [10]. mTOR activity is regulated by amino acid and glucose levels and is inhibited by starvation. In the presence of sufficient nutrients supplies, mTOR phosphorylates ULK1 and Atg13, thus inactivating the ULK1 complex [10]. But when mTOR is inhibited by starvation or treatment with the drug rapamycin, the ULK1 complex is activated and nucleation of autophagic membranes can proceed. The nucleation additionally requires the Vps34/class III PI3K complex I, which phosphorylates phosphatidylinositol to create PI3P [8]. Finally, the integral membrane protein mAtg9 must be recruited to the site of phagophore nucleation [9]. The precise origin of the membranes composing the phagophore is still under debate. But PI3P-positive structures, called omegasomes, have been found to form from the endoplasmic reticulum (ER) and newly formed autophagosomes seem to escape from these [11].

2.2. Expansion

Next, the phagophore expands and finally close to form the autophagosome. Two ubiquitin-like conjugation systems are required for this expansion [12]. Analogous to ubiquitination, the ubiquitin-like proteins Atg12 and LC3 are activated by an E1-like

enzyme (Atg7) and conjugated by an E2-like enzyme (Atg10 and Atg3, respectively) to Atg5 or phosphatidylethanolamine (PE), respectively. The Atg12–Atg5 conjugate associates with membrane-bound Atg16L to form a high molecular-weight complex. Atg12–Atg5 works in an E3-like fashion for the conjugation of LC3 to PE [13] and the Atg12–Atg5–Atg16L complex determines the sites of LC3 lipidation [14]. Before LC3 can enter the conjugation pathway it has to be cleaved at its C-terminus to expose a glycine residue. This cleavage is done by the cysteine protease Atg4 and the resulting cytosolic protein is called LC3-I. The PE-conjugated LC3 is called LC3-II. LC3-II was found to mediate membrane tethering and hemifusion and likely contributes to phagophore expansion, and it might also assist the final fusion to close the autophagosome [15]. The proteins required for nucleation and expansion of autophagic membranes are retrieved from the forming autophagosome, with the exception of LC3. LC3-II is inserted into both leaflets of the forming autophagosome. Whereas LC3-II on the outer leaflet is retrieved through delipidation by Atg4, LC3-II on the inner leaflet remains bound to autophagic membranes throughout the pathway and thus serves as an autophagic marker protein.

2.3. Maturation

After its final closure, the autophagosome matures by fusion with endocytic compartments, creating amphisomes [16], but autophagosomes may also fuse directly with lysosomes. Both the coat protein complex I (COPI) and the endosomal sorting complex required for transport (ESCRT) have been shown to be required for maturation of autophagosomes [17–19]. COPI is found at early endosomes which function as a sorting station for endocytic cargo, whereas ESCRTs are required for formation of multivesicular bodies (MVB) and sorting of endocytic cargo targeted for lysosomal degradation into MVBs. The fusion of autophagosomes with both of these endocytic compartments supports a stepwise fusion model or vesicle maturation [20], where each fusion step delivers components facilitating further fusion steps (Fig. 1). As described above, the Vps34/class III PI3K complex I is involved in nucleation of autophagic membranes, but Vps34 can also engage in a related complex, Vps34/class III PI3K complex II, which regulates the maturation of autophagosomes [8]. Moreover, the endosomal PI3P 5-kinase PIKfyve, has been shown to be involved in this maturation [21,22], indicating that also endocytic membrane lipids are important for proper autophagosome maturation.

Autophagosomes can form all over the cell cytoplasm, but must traffic to the perinuclear area to fuse with lysosomes, a process shown to depend on microtubules and dynein [23–25]. This trafficking is especially important in neurons, where the site of autophagosome formation might be located far from the lysosomes. Autophagosomes or amphisomes tether, dock and fuse with lysosomes. Among others, the class C Vps complex, as well as Rab7 and Rab11 are involved in the fusion of autophagic vesicles with lysosomes [26–29]. Recently, the Rab7-, PI3P- and LC3-binding protein FYCO1 was found to promotes microtubule plus end-directed transport of autophagosomes [30], thereby connecting transport of autophagosomes to the fusion with lysosomes.

3. Aggrephagy – selective autophagy of protein aggregates

3.1. Protein aggregates

Commonly observed human neurodegenerative diseases can be subdivided into groups based on the predominant type of aggregate-prone protein, such as tauopathies, synucleinopathies, polyglutamine-containing inclusions or TAR DNA binding protein-43 (TDP-43) proteinopathies. Moreover, aggregates are often structured (amyloid) or amorphous and are typically composed of insoluble proteins that can be hyperphosphorylated and crosslinked. Aggregate-prone proteins are thought to form microaggregates that can be combined and deposited into larger aggregates of various size and structure. In mammalian cells, aggregated proteins can be trafficked by microtubule-dependent retrograde transport to a perinuclear site of aggregate deposition called the aggresome (Fig. 2). It has been proposed that aggresomes could be a long-term storage solution for misfolded proteins waiting to be degraded by autophagy [31] and their formation could enhance the efficiency and selectivity of autophagic degradation of aggregated proteins. However, not all kinds of protein inclusions or aggresomes can be degraded by autophagy [32].

Aggregate-prone proteins are associated with cytotoxicity. In addition to no longer performing their wild-type function, the observed toxicity is thought to be a gain of function of these mutant proteins [33]. The exact nature of the cytotoxic species is still debated. Longer polyglutamine tracts in the aggregate-prone protein huntingtin correlate with earlier onset of the associated disease and higher aggregate propensity of mutant proteins [34]. This has been taken as proof that it is the aggregates that are the cytotoxic species. Others argue that soluble oligomers or small oligomers are more toxic and that aggresome or inclusion body formation protects the cells from these toxic species [35,36].

Whether entire protein aggregates are directly degraded by autophagy, or if autophagy rather degrades soluble aggregate precursors is still debated. Autophagy has also been implicated in the dissolution of larger neuronal inclusions into smaller aggregates [37]. If soluble aggregate precursors are removed by autophagy, then the aggregates could also disappear because the equilibrium would be shifted away from aggregate formation towards aggregate dissolution [33]. However, protein aggregates larger than 1 μm have also been observed inside autophagic membranes [38], indicating that the autophagic machinery can sometimes degrade entire aggregates. In this case, it is not known whether the aggregates are sequestered by preexisting autophagic membranes or if the autophagic machinery is recruited to the aggregates to mediate local assembly of autophagic membranes around the aggregate.

3.2. Selective autophagy

During starvation, autophagy is induced to recycle cellular components and support cell survival until nutrients are replenished. Therefore starvation-induced autophagy is thought to be a non-specific process, randomly sequestering cytoplasmic components [39]. However, autophagy also proceeds at basal levels, performing important quality control functions by removing damaged organelles and other substrates targeted for lysosomal degradation. It has recently become evident that this basal autophagy involves selective recognition of the autophagic cargo. The autophagic degradation of mitochondria has been termed mitophagy, whereas autophagic degradation of intracellular pathogens is known as xenophagy. For autophagy-mediated clearance of protein aggregates, the term aggrephagy was coined by the group of Seglen and co-workers [40]. Selective autophagy pathways are also present in yeast. Among these, the cytoplasm to vacuole targeting (Cvt) pathway is a biosynthetic pathway that delivers the hydrolase aminopeptidase I to the vacuole. Although not present in mammals, the Cvt pathway can serve as a model for selective autophagy also in higher organisms [41]. The key factors are cargo receptors that connect the cargo to the core autophagy machinery. Also in mammalian cells, the specific targeting of mitochondria, pathogens or protein aggregates raises the question of how the autophagic machinery recognizes these substrates. At least part of the answer lays in the recent discovery of proteins functioning

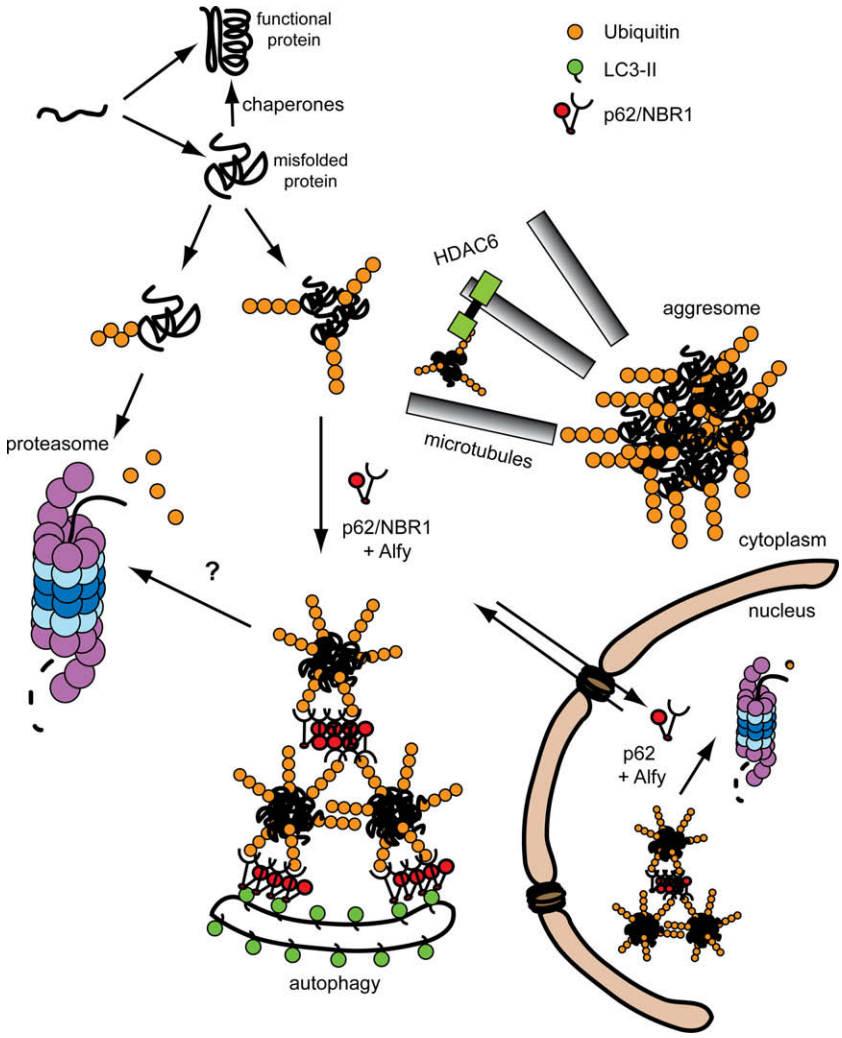

Fig. 2. *Degradation of protein aggregates by autophagy*. Newly synthesized proteins normally fold into functional proteins. However, in the case of misfolding, chaperones can sometimes help refold the protein into its native conformation, but misfolded proteins are often targeted for degradation. K48-linked ubiquitination targets the misfolded protein for degradation by the proteasome. There the proteins are unfolded to pass through the narrow pore of the barrel-shaped proteasome and the ubiquitin moieties are recycled. Misfolded aggregate-prone proteins can also be degraded by autophagy or deposited at the aggresome. These two options are associated with K63-linked ubiquitination. For transport to the aggresome, the ubiquitinated proteins are recognized by HDAC6 and transported along the microtubules in a dynein-dependent manner. The aggresome thus protects the cell from the toxicity of aggregate-prone proteins and possibly facilitates the autophagic degradation of these proteins. For degradation by autophagy, the autophagy receptors p62 and NBR1 bind to ubiquitinated proteins and self-polymerize through their PB1 domains to form p62 bodies. p62 interacts with the autophagic protein LC3 through its LIR and then, in collaboration with the large PI3P-binding protein Alfy, promotes the degradation of these protein aggregates. p62 and Alfy are also found in PML bodies in the nucleus and shuttle between the nucleus and the cytoplasm. In the nucleus, p62 is proposed to recruit proteasomes to nuclear aggregates to facilitate their degradation.

as autophagy receptors, linking the autophagic machinery to its substrates. These autophagy receptors contain an LC3-interacting region (LIR), mediating interaction with Atg8/LC3 family members (LC3/GABARAP/GATE-16) and a domain recognizing the specific substrate. Such autophagy receptors have been identified for the degradation of ubiquitinated bacteria (NDP52) [42] and mitochondria (NIX) [43], as well as for ubiquitinated protein aggregates (p62 and NBR1, described below) [44–46].

In addition to these aggrephagy-specific autophagy receptors, aggrephagy relies in general on the same molecular core machinery as non-specific starvation-induced autophagy. Neuron-specific Atg5 or Atg7 knock-out mice accumulate ubiquitinated cytoplasmic inclusion bodies [47,48] and studies in flies have shown that ubiquitinated and p62-positive aggregates accumulate in the absence of the Vps34/PI3K regulator Vps15 or Atg8/LC3 [49–51]. Likewise, siRNA-mediated clearance of Vps34/PI3K itself or its complex partner Beclin 1, inhibited autophagic clearance of an aggregate-prone model protein and the same effect was observed by treating these cells with Vps34/PI3K inhibitors [52]. In summary, the Atg5 and LC3 conjugation systems, as well as the Vps34/PI3K complex are required for autophagic degradation of protein aggregates. However, autophagic clearance of an aggregate-prone model protein can proceed even in the presence of active mTOR [52], indicating that aggrephagy is regulated differently than starvation-induced autophagy. For instance has insulin signalling, known to activate mTOR, been shown to lead to clearance of aggregate-prone proteins [52].

3.3. p62 and NBR1

p62 and neighbour of BRCA1 gene 1 (NBR1) have a very similar domain structure, with NBR1 being the larger of the two [53]. Both p62 and NBR1 are themselves substrates of autophagy and are continuously degraded [44–46]. This requires their LIR motif, which interacts with LC3 and explains how p62 and NBR1 function as cargo receptors for autophagy. Their recognition of ubiquitinated protein aggregates is mediated through the C-terminal ubiquitin-associated domain (UBA). Finally, they both contain an

oligomerization domain (PB1) through which they can homo- or hetero-oligomerize or interact with atypical protein kinase C isoforms and other binding partners [53]. In addition to being a cargo receptor for protein aggregates, p62 has been proposed to act as a cargo receptor for other ubiquitinated substrates, like ubiquitinated midbody remnants after mitosis [54] and ubiquitinated intracellular bacteria [55]. Besides its role in autophagy, p62 acts as an important scaffold or adaptor protein in other signalling pathways, like NF-κB signalling [56] and Nrf2-induced expression of anti-oxidative response genes [57].

p62 (and NBR1) is required for the formation of ubiquitinated protein aggregates (Fig. 2), also called p62 bodies, sequestosomes or aggresome-like inducible structure (ALIS) [45,58,59] mediated by its self polymerization and ubiquitin binding. These structures contain misfolded ubiquitinated proteins and can be experimentally induced by addition of the translational inhibitor puromycin [60]. Sequestration of misfolded proteins into such aggregates might prevent the misfolded, often toxic proteins from harming other cellular proteins until they become degraded by autophagy or the proteasome [59]. As described above, inhibition of autophagy in mouse brain or liver by knockout of the *Atg5* or *Atg7* genes, causes ubiquitinated protein inclusions to accumulate [47,48,61]. $Atg7^{-/-}/p62^{-/-}$ mice have much less ubiquitinated protein inclusions than $Atg7^{-/-}$ mice, showing that p62 is involved in formation of the observed aggregates [62]. This is probably because in the absence of autophagy, p62 accumulates and sequesters ubiquitinated proteins through its UBA domain, including those that would normally be degraded by the UPS [63]. Interestingly, the combined removal of *p62* and *Atg7* in hepatocytes and neurons attenuated liver impairment caused by autophagy deficiency, but had little effect on neurodegeneration. *p62* knockout mice show normal autophagy and do not accumulate protein inclusions compared to wild-type controls [62]. These effects might seem to contradict the role of p62 in aggregate clearance, but can be explained by its aggregate-promoting role. The lack of a significant phenotype in these mice can also be explained by the redundant function of NBR1. Corresponding to autophagy-deficient mice, autophagy-deficient fruit flies show accumulation of ubiquitinated protein aggregates [50,51,64]. These aggregates are positive for the *Drosophila* orthologue of p62, Ref(2)P, and the aggregates disappear in autophagy and *Ref(2)P* double mutants [51].

p62 is also a common component of ubiquitinated protein inclusions found in human diseases, including Lewy bodies (Parkinson's disease (PD)), neurofibrillary tangles (Alzheimer disease (AD)), Huntingtin aggregates (HD) and Mallory bodies (alcoholic and nonalcoholic steatohepatitis) [65,66]. Immunostaining of p62 on histological samples can be employed as a diagnostic marker for human degenerative diseases associated with protein aggregates [67] and it will be interesting to learn whether accumulation of p62 in these diseases is associated with dysfunctional autophagy.

3.4. Alfy

In addition to p62 and NBR1, the large protein Alfy (autophagy-linked FYVE protein, also known as WDFY3) is involved in degradation of protein aggregates by autophagy (Fig. 2). Alfy becomes recruited from the nucleus to cytoplasmic ubiquitinated protein aggregates also containing autophagic membrane markers upon cellular stress like starvation and proteasome inhibition [68]. Alfy was recently shown to interact directly with p62 and, similar to p62 and NBR1, be required to recruit ubiquitinated proteins into aggregates that become degraded by autophagy [69]. Alfy is a large scaffolding protein containing several domains which can facilitate recruitment of the autophagic machinery to the protein aggregates; a PH domain, a p62-interacting BEACH domain [69], a PI3P-binding FYVE domain [68] and WD40-repeats engaged in interaction with Atg5 [38]. In line with these results, it was recently found that Alfy is recruited to intracellular inclusions where it scaffolds a complex containing p62 and the autophagic effectors Atg5, Atg12, Atg16 and LC3. Whereas loss of Alfy was found to inhibit inclusion clearance, its overexpression caused elimination of aggregates in an Atg5-dependent manner, and led to protection in a neuronal and Drosophila model of polyglutamine toxicity [38]. These results corroborate previous studies reporting that fruit flies mutant for the Alfy homologue, *blue cheese* (Bchs), show reduced life span due to accumulation of ubiquitinated protein aggregates in the brain [70]. It is not known if Alfy can recognize the ubiquitinated aggregates directly or if it is recruited through its interaction with p62.

3.5. HDAC6

Autophagy and the UPS both target misfolded proteins for degradation. Proteolytic cross-talk clearly exists between the two pathways, shown by autophagy acting as a compensatory degradation system when the UPS is blocked [71,72]. Histone deacetylase 6 (HDAC6), a tubulin deacetylase that binds ubiquitin via its C-terminal BUZ-domain, was shown to mediate this effect, probably by bringing proteins that would otherwise accumulate to the autophagic machinery. Overexpression of HDAC6 in a fly model of neurodegenerative disease (Spinobulbar muscular atrophy caused by polyglutamine repeat expansion in the androgen receptor), accelerated the degradation of the mutant aggregate-prone protein by autophagy and protected the flies from neurotoxicity [71]. HDAC6 is required for the formation of aggresomes by transporting misfolded proteins to the microtubule-organizing centre (MTOC) along microtubules through interaction with dynein [73,74] (Fig. 2). HDAC6 is also thought to mediate the transport of components of the autophagic machinery to the aggresome, thus bringing the machinery close to its substrates [72]. Recently, HDAC6 was also found to promote the creation of an actin network that enhances the fusion of autophagosomes and lysosomes [75]. This effect was specific to basal quality-control autophagy and dispensable for starvation-induced autophagy.

4. Regulation of autophagic degradation of protein aggregates

The degradation of aggregate-prone proteins by autophagy can be regulated both at the level of the autophagic machinery and at the level of the aggregates, but not much is known about this regulation. As mentioned above, aggrephagy seems to proceed independent of mTOR signalling and might be regulated by insulin signalling [52]. Reactive oxygen species (ROS), known to accumulate by age, has been found to inactivate Atg4, thus inhibiting LC3 delipidation and potentially promoting autophagy [76]. However, although a clear correlation was found between reduced autophagy levels, formation of ubiquitinated protein aggregates and sensitivity to oxidative stress in *Drosophila* [50], further studies are required to elucidate how ROS, as well as the p62-mediated Nrf2-induced anti-oxidant response [57], regulates aggrephagy and disease progression.

Several proteins of the autophagic machinery localize to the nucleus and undergo nucleocytoplasmic shuttling to exert their function in the cytoplasm. Very little is known about how this nucleocytoplasmic shuttling affects autophagy and no thorough study of which of the autophagy associated proteins rely on nucleocytoplasmic shuttling have been performed. Concerning the aggregates, various post-translational modifications, like ubiquitination, acetylation and phosphorylation, have been shown to specifically enhance the degradation of the aggregate-prone proteins by autophagy.

4.1. Nucleocytoplasmic shuttling

The nucleus and cytoplasm are separated by the nuclear envelope. Small water-soluble molecules and proteins smaller than ~40 kDa can freely diffuse between the compartments through the nuclear pores, whereas larger proteins must actively be transported. Nuclear import is usually mediated by importin-a binding to a nuclear localization signal (NLS) and nuclear export by exportin-1/CRM1 binding to a nuclear export signal (NES) of the shuttling protein. The assembly and disassembly of these complexes is mediated by the GTP/GDP-bound status of the small GTPase Ran [77].

The autophagic proteins found to undergo nucleocytoplasmic shuttling so far are Beclin 1, diabetes- and obesity-regulated gene (DOR), p62 and Alfy. The former two are required for starvation-induced autophagy, whereas the latter two are implicated in more specific forms of autophagy as described above. The first autophagic protein shown to rely on nucleocytoplasmic shuttling was Beclin 1 [78]. Beclin 1 is part of the PI3K complex and has a tumor suppressive function [79]. It contains a NES motif which is required for Beclin 1 to promote starvation-induced autophagy and inhibit tumorigenicity [78]. Recently also DOR, a reported nuclear cofactor of thyroid hormone receptors, was implicated in autophagy [80]. Upon starvation, DOR exits the nucleus to interact with LC3 family members in the cytoplasm and promote autophagy [80]. Mutation of the DOR NES motif restricted DOR to the nucleus even under stress conditions and blocked induction of autophagy by rapamycin.

Alfy localizes to the nucleus and nuclear envelope in HeLa cells, but is redistributed to protein bodies in the cytoplasm upon starvation or proteasomal inhibition [68]. In contrast to the predominant nuclear localization of Alfy, p62 is found to shuttle continuously between the nucleus and the cytosol at a high rate [81]. p62 was recently found to interact directly with Alfy and to be required to recruit Alfy from the nucleus to the cytoplasmic ubiquitinated protein bodies [69]. This recruitment of Alfy from the nucleus is essential for autophagic degradation of larger complexes of ubiquitinated proteins. p62 contain two NLS and one NES motifs, and the shuttling of p62 is modulated by its phosphorylation and aggregation status. At steady state, the majority of the p62 pool is found in the cytoplasm, but this is rapidly changed by inhibition of nuclear export [81]. Interestingly, most nuclear autophagy proteins identified to date were found to localize to promyelocytic leukemia nuclear bodies (PML-NBs), especially when exportin-1/CRM1-dependent nuclear export was inhibited. PML nuclear bodies has been associated with a broad spectre of nuclear processes, as transcription, DNA repair, stress, proteolysis and apoptosis [82]. The nuclear proteasome-mediated protein degradation occurs at a subset of PML-NBs [83]. Both p62 and Alfy localize to PML nuclear bodies and are required for the recruitment of ubiquitinated proteins to these nuclear bodies [69] (Fig. 2). p62 has been suggested to facilitate proteasome recruitment to these sites and thereby help the degradation of nuclear ataxin-1 aggregates [81] (Fig. 2). However, it cannot be excluded that p62 and Alfy export ubiquitinated substrates to the cytoplasm for degradation by the autophagic machinery. It will also be interesting to learn whether the nuclear autophagic proteins undergo some kind of regulation or modification at the PML-NBs.

4.2. Ubiquitination

Several post-translational modifications of aggregated proteins have been found to enhance their degradation through autophagy. First, protein aggregates targeted by autophagy are generally ubiquitinated. Ubiquitin chains come in different flavours depending on through which lysine residue the ubiquitin moieties are linked. K48-linked ubiquitin chains are a classical signal for degradation via the UPS. It is not clear if autophagic degradation is signalled through a specific ubiquitin-link. However, p62 and NBR1 have been shown to preferentially recognize K63-linked ubiquitin chains [46,84,85] and inclusions labelled with K63-linked ubiquitin chains have been associated with autophagic degradation [86]. Furthermore, HDAC6 also preferentially binds K63-linked ubiquitin chains in vivo [87]. p62 is also known to interact with the E3 ubiquitin ligase TRAF6, which catalyzes K63 polyubiquitination [56] and it is thus likely that TRAF6 could contribute to the ubiquitination of protein aggregates degraded by autophagy. Thus, cargo modified with K63-linked ubiquitination might therefore be preferentially targeted to the autophagic degradation pathway. Actually, ubiquitination alone might be sufficient for autophagic targeting via p62. Indeed, peroxisomes or a cytosolic model protein (red fluorescent protein) tagged with ubiquitin became targeted for autophagic degradation in a p62-dependent manner [88].

4.3. Acetylation

Acetylation of mutant aggregate-prone huntingtin enhances its degradation by autophagy [89]. In contrast, mutant huntingtin that is resistant to acetylation strongly accumulates in the expressing cells. Huntingtin is actually a nuclear protein and must be exported to the cytoplasm to be degraded by autophagy. The described acetylation is performed by nuclearly localized CREB-binding protein (CBP), but it is not clear if the acetylation enhances the nuclear export of the mutant huntingtin or its actual degradation by the autophagic machinery in the cytoplasm. It will be interesting to learn if also other proteins can be targeted for lysosomal degradation through acetylation.

4.4. Phosphorylation

In addition to acetylation, it was recently reported that phosphorylation by the IKK complex also enhances degradation of mutant aggregate-prone huntingtin [90]. Phosphorylation of huntingtin was found to regulate other post-translational modifications like ubiquitination, SUMOylation and acetylation. The increased degradation of phosphorylated huntingtin proceeded both through the proteasome and the lysosome. However, in this study IKK-mediated phosphorylation was more efficient for wild-type than mutant huntingtin, suggesting that the phosphorylation-driven clearance of mutant huntingtin is reduced. Previously it has been found that phosphorylation of mutant aggregate-prone polyglutamine proteins (ataxin-1, ataxin-3 and huntingtin) also regulate their ability to get cleaved, aggregate and cause neurodegeneration [91–96].

5. Autophagic degradation of aggregate-prone proteins in disease

Basal autophagy is especially important in post-mitotic cells like neurons and hepatocytes, which cannot get rid of their waste through cell division. As previously discussed, inhibition of autophagy in mouse brain or liver by knockout of the *Atg5* or *Atg7* genes, causes ubiquitinated protein inclusions to accumulate [47,48,61]. Importantly, brain-specific suppression of autophagy causes neurodegeneration even in the absence of aggregate-prone disease-associated mutant proteins, showing that basal quality-control autophagy is important to prevent neurodegeneration [47,48]. Several neurodegenerative phenotypes are associated with accumulation of protein inclusions, including polyglutamine diseases, PD, frontotemporal dementia (FTD) and AD. Autophagy has been implicated in all of these diseases.

5.1. Polyglutamine diseases

There are nine different polyglutamine diseases, all characterized by abnormal expansion of a polyglutamine tract in a specific protein. The most common and best studied of these, HD, is caused by aggregate-prone mutant forms of the protein huntingtin having a polyglutamine tract of more than 37 glutamines. The disorder involves neuronal loss in the striatum and cortex leading to gradual loss of voluntary movement coordination and eventually death of the patient. Longer polyglutamine tracts in huntingtin correlate with earlier onset of the disease and higher aggregate propensity of the mutant proteins [34]. Mutant full-length huntingtin and N-terminal fragments thereof aggregate and become substrates for autophagy [97–99]. Moreover, expression of aggregate-prone mutant huntingtin has been found to increase autophagy in HD mouse models and patients [100–102]. This induction of autophagy can be explained by decreased mTOR activity and eIF2α phosphorylation [103,104]. Importantly, autophagy is specifically important for the degradation of aggregate-prone mutant huntingtin and not wild-type soluble huntingtin [97]. Another aggregate-prone polyglutamine protein, ataxin-3, causing spinocerebellar ataxia type 3 (Machado-Joseph disease), was also found to be degraded by autophagy [105].

Another type of ataxia (lack of muscle control or coordination) is caused by the polyglutamine disease protein ataxin-1, which forms aggregates in the nucleus. Polyglutamine expanded versions of ataxin-1 are the etiological factors of spinocerebellar ataxia 1 (SCA1). If and how nuclear aggregates are degraded by autophagy is currently debated in the field. Proteasomes are present in the cell nucleus, but lysosomes are not. Huntingtin is also found in the nucleus, and aggregate-prone mutant versions of huntingtin must be exported to the cytoplasm to be degraded by autophagy. As described above, this might be regulated by acetylation and phosphorylation of huntingtin [89,90]. Autophagy has been shown to eliminate cytoplasmic polyglutamine-containing aggregates more efficiently than nuclear aggregates [106] and this differential efficiency of degradation could explain why nuclear aggregates are more cytotoxic than cytoplasmic ones.

5.2. Parkinson's disease

PD results from loss of dopamine-producing neurons causing tremor, rigidity and impaired balance and coordination. Autophagy has been found to be elevated in PD patient neurons [107]. PD is characterized by the formation of neuronal aggregates called Lewy bodies. The major protein constituent of these Lewy bodies is α-synuclein, which can be degraded by the proteasome, macroautophagy and CMA [108,109]. A30P and A53T mutations of α-synuclein cause familial forms of PD [110]. These mutant proteins were shown to bind the CMA receptor lysosomal associated membrane protein type 2A (LAMP-2A) on the lysosomal membrane, blocking their uptake by CMA and inhibiting their own degradation as well as that of other substrates [109]. α-Synuclein undergoes different types of posttranslational modifications, including dopamine-modification, which also inhibits the CMA-mediated degradation of itself and other substrates [111].

5.3. FTD and amyotrophic lateral sclerosis (ALS)

Dysfunction of the autophagic machinery can also cause neurodegenerative disease. FTD is the most common form of presenile dementia after AD, characterized by shrinking of the frontal and temporal anterior lobes of the brain and either tau or ubiquitin neuropathology [112]. ALS is a fatal disease characterized by death of motor neurons, resulting in the ability of the brain to control voluntary muscle movement being lost. Both diseases are associated with ubiquitinated protein inclusions, formed by proteins like tau, TDP-43 or SOD1, that also contain p62 [113]. Rare mutations in the ESCRT subunit CHMP2B are associated with FTD and ALS in patients [114,115]. Individual deletion of several ESCRT components resulted in accumulation of non-degradative autophagosomes in nematodes, flies or mammals [116]. Additionally, reduced ESCRT function in a fly model for HD aggravated polyglutamine-induced neurotoxicity [21]. Furthermore, accumulation of ubiquitinated protein aggregates was observed in cortical neurons of mice [19] or in HeLa cells [17], expressing mutant CHMP2B. This mutation corresponds to a rare patient mutation found in FTD patients, underlining the importance of functional MVBs for proper autophagic maturation and showing that a functional ESCRT machinery is physiologically important for autophagy in mammals.

Point mutations of the p150 subunit of dynactin have also been described in ALS patients [117]. Dynactin is essential for dynamin-dependent transport on microtubules and as discussed above, dynein function is important for autophagosome–lysosome fusion. Dyneins are also mutated in some forms of motor neuron disease and protein aggregation is present in some of these conditions, possibly as an effect of compromised autophagy [33]. Indeed, mutations in the dynein machinery impair autophagic clearance of aggregate-prone proteins and enhance their toxicity in fly and mouse neurodegenerative models [118].

Finally, mutations in the hexameric chaperone p97/VCP cause a dominantly inherited disease known as inclusion-body myopathy with Paget's disease of the bone and frontotemporal dementia (IBMPFD), characterized by nuclear and cytoplasmic aggregates in the brain and muscles of the patient [119]. The appearance of aggregates has previously been thought to result from compromised shuttling of ER associated degradation (ERAD) substrates to the proteasome [120]. However, p97/VCP was recently implicated in autophagy and compromised autophagy might contribute to the symptoms observed in IBMPFD [121]. Interestingly, mutations in p62 are also known to cause Paget's disease of the bone, although it is not known if this is linked to the role of p62 in autophagy or rather its involvement in other signalling pathways [53].

5.4. Alzheimer disease

Autophagosomes and autolysosomes have been found to accumulate in dystrophic neurites of AD patients [122]. AD is the most common form of dementia, starting with memory loss and confusion, gradually leading to behaviour and personality changes and eventually severe loss of mental function. AD is associated with formation of extracellular plaques consisting of aggregated amyloid-β (Aβ) peptide and intracellular neurofibrillar tangles composed of hyperphosphorylated tau. The Aβ peptide is formed by cleavage of the membrane protein amyloid precursor protein (APP) by the enzymes β- and γ-secretase. γ-Secretase and Aβ itself has been found within autophagosomes, suggesting autophagosomes to be a site of Aβ production [123]. Indeed, Aβ levels increase upon induction of autophagy. The produced Aβ would normally be degraded in the lysosome [124], but the increased formation of autophagosomes combined with impeded turnover of these vesicles creates favourable conditions for Aβ accumulation and appearance of AD symptoms [123].

5.5. Mallory-Denk bodies (MDBs) in chronic liver diseases

Diseases that involve ubiquitinated protein aggregates are not only neurodegenerative. There are also examples of ubiquitinated protein aggregates found in muscle and liver disease. In the liver, MDBs are found in patients with diverse chronic liver diseases, including alcoholic and non-alcoholic steatohepatitis [125]. MDBs

are intermediate filament (IF)-related aggregates. IFs are components of the cytoskeleton composed of different types of proteins and are involved in the maintenance of cell shape, locomotion and intracellular trafficking. Pathophysiologically, aggregation of several different IF subunits is associated with human disease [126]. IFs are also regularly found in aggresomes [31]. MDBs are composed of aggregated abnormal keratins and p62 is found to be required for their formation in cell culture [127]. By immunohistochemistry it was shown that MDBs are also positive for NBR1 [46]. Autophagy was found to be increased in a mouse model of MDBs and further induction of autophagy by rapamycin increased the clearance of these aggregates [128].

In summary, the different types of disease-associated protein aggregates mentioned are substrates for autophagy. Some aggregates are also formed due to autophagic dysfunction. The degradation of these disease-associated aggregates depends on p62 and the core autophagic machinery. Consequently, by learning more about the mechanisms of autophagic degradation of one type of aggregates, the knowledge can likely be applied to several types of aggregates.

6. Treating protein aggregate associated diseases by manipulating autophagy?

The neurodegenerative phenotype of diseases involving aggregate-prone proteins seems to depend on the continuous presence of the mutant protein. Neurodegenerative symptoms in a mouse HD model have been shown to be alleviated by direct RNA interference targeting the mutant huntingtin [129]. Interestingly, halting expression of the mutant aggregate-prone protein in symptomatic mice was found to cause disappearance of the inclusions and a complete reversal of the neurodegenerative symptoms in mouse models of HD and SCA1 [130,131]. Therefore it is postulated that removing the aggregate-prone proteins would cure patients of neurodegenerative symptoms. The removal could happen either by stopping the expression of the aggregate-prone protein or enhancing its degradation. As we have seen, autophagy can degrade protein aggregates. Thus, induction of autophagy could be envisaged as a treatment option for patients suffering from protein aggregate associated diseases, provided that there is no downstream block in the autophagy pathway. If such a block at the lysosome level exists, increasing autophagy may actually aggravate the condition.

Expression of autophagy genes has also been seen to decrease with ageing, simultaneous with an increase in intracellular, ubiquitinated protein aggregates [50,132]. Most protein aggregate associated diseases are also late-onset [133]. The decrease in autophagic activity with age would probably aggravate the disease state by reduced clearance of the aggregate-prone proteins. Fruit fly experiments showed that genetic promotion of autophagy in the brain of adult flies reduced the amount of oxidized and ubiquitinated protein species [50]. These effects were also accompanied by increased fly lifespan.

Gene therapy in humans is not an immediate option for treating neurodegenerative diseases by inducing autophagy. However, autophagy can also be induced by drugs. The mTOR inhibitor rapamycin is frequently experimentally used to induce autophagy and is currently in clinical use, although not specifically to induce autophagy [33]. Ravikumar and colleagues showed that mTOR inhibition induced autophagy and reduced toxicity of mutant aggregate-prone huntingtin in fruit fly and mouse HD models [103]. Actually, mTOR inhibition through rapamycin treatment alleviates toxicity of different aggregate-prone proteins, including those found in polyglutamine diseases, PD and some forms of FTD [134]. The treatment with rapamycin reduces the levels of both soluble and aggregated proteins. An additional cytoprotective effect of rapamycin is that it protects the cells against apoptotic insults, probably through increased removal of mitochondria by mitophagy [135]. However, mTOR controls many other pathways in addition to autophagy and long-term use of rapamycin brings certain side effects, like immunosuppression (this effect is used to prevent rejection of the transplant in renal transplant patients). Thus inducing autophagy without inhibiting mTOR would be desirable. mTOR-independent autophagy pathways and drugs have been described [136,137]. For instance, lithium induces autophagy by reducing intracellular inositol levels [138]. Additionally, increased efficiency of autophagy induction can be expected by combining mTOR inhibition through rapamycin analogues with mTOR-independent small molecule enhancers [139]. However, keeping in mind the problems associated with accumulation of autophagosomes in AD, it is important not only to induce autophagy, but to ensure proper flux throughout the autophagic pathway.

In addition to general activation of the autophagy pathway, specific degradation of aggregate-prone proteins could be envisaged by enhancing the posttranslational modifications that promote their degradation. For instance, enhancing acetylation of mutant huntingtin improves its degradation by autophagy and reverses the toxic effects in primary striatal and cortical neurons [89]. HDAC inhibitors might be used to enhance acetylation in general. We are, however, currently not aware of any direct agent to enhance the specific acetylation of mutant huntingtin, but discovery of drugs that enhance this or other posttranslational modifications that promote clearance of aggregate-prone proteins could benefit future patients.

7. Concluding remarks

From the initial idea of a random non-selective process, autophagy has emerged as a collection of many selective pathways targeting specific cargo for degradation. Moreover, autophagy has been established as an alternative pathway to the UPS for degradation of ubiquitinated proteins, specializing in the degradation of aggregate-prone proteins. This degradation requires the autophagy receptors p62 and NBR1 that are also found to localize to various protein aggregates associated with human diseases. In general, inducing autophagy seems to promote aggregate clearance and alleviate the cytotoxic effects of aggregate-prone proteins across many of these diseases, although imbalance in the autophagic flux can be harmful. To develop better tools to enhance degradation of aggregate-prone proteins by autophagy and potentially employ these in therapy, we need greater insight into the mechanisms governing how these proteins are targeted for autophagic degradation.

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